REMARKS

The Examiner has made final the restriction requirement and has limited the present examination to claim 3. Applicants have amended the claims accordingly with traverse and request reconsideration of the restriction requirement for the reasons stated below.

Applicant has amended the specification to provide information on the parent application which has issued as US Patent 6,811,782. As presented claim 3 adopts language that has been allowed and issued.

Claims 7 and 10 are amended to depend on claim 3. The claims now pending are claims 3, 7 and 10.

RESPONSE

The Examiner has limited the present examination to claim 3 contending that the claims of the present invention are directed to a protein. Applicant wish to point out that the claims of the present application is a synthetic peptide comprising a B-cell epitope and Th epitope for eliciting antibodies against IgE for the treatment of allergy. The B-cell epitope is derived form IgE-CH3 domain and is conjugated to selected Th epitopes for presenting the B-cell epitope. Thus, the present claims are directed to well defined B-cell epitopes and conjugated to well defined Th epitopes. The invention is in the field of immunonology where it is well known that there are epitopes on proteins that may be selected for its function and used to provoke a B-cell to produce antibodies to the epitope. It is well known that antibodies and epitopes fit together like lock and key. The epitope has a particular conformation and the antibody that binds to it has a particular conformation. Thus the conformation of the B-cell epitope is of particular importance.

The claims of the present invention are directed to IgE-CH3 B-cell epitopes from human IgE, dog IgE, mouse IgE, mice IgE and horse IgE (SEQ ID;NO5, 6. 7. 8 and 84). Although there are differences in the amino acid sequences they all function as a B-cell epitope to provide antibodies against IgE to prevent the cascade wherein IgE provokes the production of histamine and the attending symptoms of an allergy.

In the present case, the B-cell epitope is also cyclized with cysteines to ensure that the epitope is of a particular conformational structure. The Examiner contends that it is known that in a protein a single change of an amino acids can cause the change in the structure of a protein. This may be true in some cases. But, it is not true when applied to B-cell epitopes. Othewise, the filed of immunology would not exist.

Applicant has provided a multitude of such permutations showing how a B-cell epitope of different allelic species may have different amino acid sequences. See Table 1 and Table 3. Applicant also provided information on Th epitopes in Table 5 and how such Th epitopes may be modified in Table 6.

Firstly, a single general inventive concept is presented by this application. The concept is that there is an epitope in IgE-CH3 domain which is useful when conjugated to a promiscuous Th epitope to generate antibodies against IgE for the treatment of allergies. The epitope is defined by SEQ ID NO:5. Analogues of SEQ ID NO:5 were also presented. These are SEQ ID NO: 6, 7, 8 and 84. A comparison of the sequences for SEQ ID NOs: 5, 6, 7, 8 and 84 is presented below to show that they are analogues of each other.

SEQ ID NO:5	CGETYQSRVTHPHLPRALMRSTTKC
SEQ ID NO:6	CGETYYSRVTHPHLPKDIVRSIAKC
SEQ ID NO:7	CGEGYQSRVDHPHFPKPIVRSITKC
SEQ ID NO:8	CGYGYQSIVDRPDFPKPIVRSITLC
SEQ ID NO:84	CGETYKSTVSHPDLPREVVRSIAKC

A further comparison of these sequences will show that they correspond with a part of IgE-CH3 that is modified from humans, dog, rat, mouse and horse. See Table 1 on page 67 of the specification. These five sequences are then respectively conjugated to a promiscuous Th epitope selected from the group consisting of SEQ ID NOs: 9-12, 60-82 and 89 disclosed and set forth on Tables 5 and 6. The conjugated product may further be conjugated with an invasin domain to further improve the immunoresponse, i.e. to increase the titer of the antibodies to IgE elicited for more effective treatment of

allergies. Thus, the invention claimed and described is directed to a single general inventive concept.

Since the peptides in its different permutations are shown to be effective to provide antibodies to IgE for the treatment of allergies, they are different embodiments of a single inventive concept. Applicants request withdrawal of the restriction requirement.

Moreover, claims 5 and 6 presents a different way of setting forth the claimed invention of a synthetic peptide and how it may be conjugated to Th epitopes with more specificity. It is not understood how presenting an invention with more specificity results in a different invention.

The Examiner has limited the examination to claim 3 directed only to a synthetic peptide antigen of about 50 to 90 amino acids comprising a helper T cell epitope, SEQ ID NO:5 and optionally SEQ ID NO:13.

Claim 3 was rejected on the following bases each of which is responded to as follows.

Rejection under 35 U.S.C. §112, second paragraph

Claim 3 was rejected for reciting "about 25 to about 29 amino acids" and "about 23 amino acids". The contention is that the term "about" renders the claim unclear and indefinite in that the term may mean 4, 11 or more amino acids.

Reconsideration is requested for the following reasons.

Firstly, claim 3 adopts language that was used, and discussed with the prior Examiner. The language was regarded as clear and definitely to those of skill in the art.

Secondly, claim 3 recites that SEQ ID NO:5 can vary by up to 4 amino acids. Thus, it is clearr and definite. It is not clear how the Examiner picked the number "11" or more.

Rejection under 35 U.S.C. §112, first paragraph

Claim 3 was rejected for lack of enablement of a synthetic peptide of "about 50 to 90 amino acids". The Examiner picked two of the synthetic peptides presented in the application SEQ ID NO 14 and SEQ ID NO:15, which had 45 or 63 amino acids and contends that there is lack of enablement of about 50 to about 90 amino acids.

Applicant would like to point to Table 4A which presents 18 peptide conjugates as different embodiments of the claimed invention. SEQ ID NO: 20 contains 126 amino acids, SEQ ID NO :27 contains 79 amino acids; SEQ ID NO:87 contains 65 amino acids, SEQ ID NO:88 contains 57 amino acids, SEQ ID NO:19 contains 66 amino acids, SEQ ID NO:90 contains 42 amino acids, and SEQ ID NO:91 contains 61 amino acids. Thus there is ample support for "about 50 to about 90 amino acids."

The Examiner also contends that the specification shows test results for SEQ ID NO:14 and SEQ ID NO:15 and thus does not enable full scope of claim 13. Reconsideration is requested.

It appears that only Table 3 was looked at. Applicant would like to point to Table 2 which provided results of antibodies produced which were cross-reactive with human IgE. There are 15 peptide constructs in accordance with the invention claimed that provided very good cross reactivity. Table 4B also provided results of 6 peptide constructs according to the claimed invention which showed cross reactivity with two of the peptides showing prevention of histamine inhibition. Also Table 7 provided results for inhibition of passive cutaneous anaphylaxis using SEQ ID NO:25.

Thus, it is believed that the claimed invention is enabled. The patent law does not required that each and every embodiment of the invention be tested and the data be presented. Applicant has tested sufficient of the embodiments to show that the invention as claimed is enabled and supported. However, applicant should not be limited by the test results. The test results are illustrative.

Moreover, it is not proper to limit the examination by a restriction requirement, then examine the application for test results limited to the embodiment so restricted. The data in support of the invention as a whole should be considered. Applicant is only

required to provide sufficient information and test data to a person of skill in the art to show that the invention as claimed is enabled.

The Examiner points to the unpredictability of protein chemistry. However, the claims are not directed to proteins. The claimed invention is directed to a synthetic peptide comprising of segments with known functions, that of a B-cell epitope and that of a Th epitope. Applicant has developed the B-cell epitope for IgE-CH3. The Th epitopes are well known promiscuous Th-epitopes and those derived therefrom by the Applicant. Applicants work is based on known principles in immunology and the field of synthetic peptides. See Synhetic Peptides as Antigens, Ciba Foundations Symposium 119, 1986, pp. 279-291, John Wiley and Sons, a copy of which is enclosed.

Enclosed herewith is copy of Wang et al, Vaccine, 2003, 21:1580-1590. The article shows that the invention claimed has been reviewed by its peers, people of skill in the art, who found the invention to be of merit. In addition, the invention is in the process of being commercialized and is in clinical trials. The publication further shows that to persons of skill in the art the invention as claimed is enabled.

Withdrawal of the rejection on these grounds should be withdrawn.

CONCLUSION

It is believed that the invention claimed is patentable. The believe is supported by the issuance of US Patent 6,811,782. It is hoped that the submission of a divisional application does not raise any questions that had previously been settled.

AUTHORIZATION

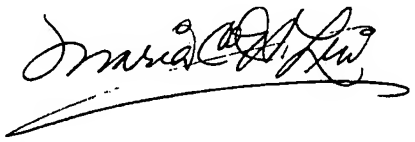
The Commissioner is hereby authorized to charge any additional fees which may be required for consideration of this Amendment to Deposit Account No. 13-4500, Order No. 1151-4153US2.

27123
↑CUSTOMER NUMBER↑

Docket No. 1151-4153US2

In the event that an extension of time is required, or which may be required in addition to that requested in a petition for an extension of time, the Commissioner is requested to grant a petition for that extension of time which is required to make this response timely and is hereby authorized to charge any fee for such an extension of time or credit any overpayment for an extension of time to Deposit Account No. **13-4500**, Order No. 1151-4153US2.

Respectfully submitted,



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Ciba Foundation Symposium 119
Synthetic peptides
as antigens

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Final general discussion

Reactivity of anti-peptide antibodies with the parent proteins

Ada: A number of topics remain for further general discussion. They include the proportion of monoclonal antibodies which react to the different types of epitopes in a polymeric protein, ranging from linear determinants to quaternary structures; the roles of high versus low affinity antibodies; and antigen presentation with T-dependent and T-independent antigens. But first we should discuss the question of antibodies to linear peptides reacting with the native proteins. This is a crucial aspect if peptides are to be used as the basis of future vaccines. Members of the group may be prepared to quote from their own experience, as I have the feeling that there is considerable variation in the literature.

Geysen: In our approach, we first show that a particular epitope was recognized by analysing the anti-protein response; the corresponding peptide is then synthesized, and the subsequent anti-peptide response has in all cases reacted with the original protein. That this is so is not surprising, as there was no prediction involved in the choice of which peptide to use. More importantly, we also determine the specificity of the antibodies induced by either antigen—protein or peptide. This looks past the superficial aspect of whether the anti-peptide response recognizes the protein; that is, whether each antigen induces antibodies interacting with the same contact residues.

Skehel: In the influenza system a remarkable feature of antibodies against peptides equivalent to regions of the haemagglutinin is that although all the antibodies appear to react with the peptide used to induce them, none seems to neutralize virus infectivity, and although they interact with haemagglutinin in procedures such as immunoblotting they don't immunoprecipitate native haemagglutinin. I wonder how common that is. One would imagine from such results that attempts to use peptides as vaccines against influenza would be a waste of time.

Crumpton: Let me underline this important point by referring to some results of work carried out in Mike Waterfield's laboratory of ICRF in collaboration with José Schlessinger at the Weizmann Institute, and Axel Ullrich at Genentech (Gullick et al 1985). Fifteen peptides of EGF receptor were synthesized, coupled to KLH and used to immunize rabbits; six peptides were

located in the receptor's extracellular domain. All antisera contained high titres of antibodies against the homologous peptide, but only five peptides gave antisera which reacted with the native receptor, as judged by immunoprecipitation. All of the latter five peptides were located in the receptor's cytoplasmic domain. So there was a contrast between the responses induced by peptides from the extracellular and cytoplasmic domains, as judged by the reactivity of the antisera with the native receptor. The reason for this is not known. The most striking feature was the relatively small number (33%) of anti-peptide sera which reacted with the native EGF receptor.

Stevens: I think you are talking about peptides sequences where a structure has to be recognized in order for elicited antibodies to react with the receptor. That may be quite different from when an epitope consists of linear sequences in a soluble protein.

Our experience with making antibodies to peptides for reacting to an intact protein suggests that an antibody made to a peptide *per se* will not necessarily be of high reactivity, but if you can locate a peptide region that is on the surface of the parent protein molecule, there is a good probability of getting cross-reactivity. I don't think you necessarily have to look for the immunodominant site of the native protein to use for immunization. As Dr Sela's laboratory has shown, you can select a region that is not immunogenic in the native molecule and prepare a peptide to that region, so long as it is on the surface; after immunization with the peptide, you can obtain a high degree of recognition of the whole protein.

Geysen: It depends how you choose the peptide. If you just look at the sequence of the protein, choose a stretch which has, say, four arginines next to two glycines, and decide to make a peptide corresponding to that, the chances of the anti-peptide response reacting with the native antigen are almost zero. It comes down to how you select the peptide, which determines the frequency of finding that the anti-peptide response reacts with the protein.

Crumpton: One of the common rules that many people use for selection of such peptides is hydrophilicity.

Lerner: Many experiments have been published in which people have made anti-peptide antibodies that see proteins. There are also some which don't work. But anti-peptide antibodies are a site-directed technology with none of the circularity usually inherent in immunological procedures. There, one says: 'Let's screen for an antibody which neutralizes'. You let the selective system churn for you, until you get such an antibody. No surprise! In contrast, in the anti-peptide approach, we say: 'if I make an antibody to a certain shape at a certain point, I think it will do something'. In the absence of knowledge of the three-dimensional structure we must to some extent depend on guesswork.

Skehel: From these many peptides that work and the many that don't work, you would hope by now to have some clue about what makes them work.

Lerner: Why? Do you have a clue about how proteins fold, with only 20 amino acids?

Skehel: So the answer is that it is still a hit-and-miss process.

Geysen: It's more like marksmanship. If you are a good marksman you hit the target 99 times out of 100. If you are a very poor marksman, you never hit the target.

V. Nussenzweig: What are the rules of marksmanship, though?

Geysen: Just that some are better than others!

Sela: I would like to come back to what Richard Lerner called a paradox. What exactly are we talking about here? Some of the sequences in a protein are in a form in which they are very rigid and the related free peptides have a completely different conformation from the sequence within the native protein. The chance that such a peptide will transconform into the shape within the native protein is small. There are other regions in that protein which are more flexible and the chance that a peptide related to such a region may transconform into a shape similar to that present in the native protein is much better. I would like to stress, too, that the question is *not* whether there are cross-reacting antibodies. One should distinguish between 'reaction' and 'useful reaction', because a weak cross-reaction is not interesting for practical purposes, such as vaccination.

When you look at the proteins, my view is that those proteins that need disulphide bridges for their three-dimensional structure use them as crutches, otherwise their structure collapses readily. On the other hand, the coat proteins of viruses, for example, most of the time lack disulphide bridges, and segments of the complete protein are probably already in a shape much more similar to that in the intact molecule.

Corradin: My experience of making anti-peptide antibodies that cross-react with their native proteins is restricted to cytochrome *c*. I have made either polyclonal or monoclonal antibodies against both the C-terminus and the N-terminus, which contain many lysine residues and are flexible by definition. On ELISA, the antibodies cross-react well with native cytochrome *c*. The antibodies cross-react also with other peptides on ELISA. If we determine whether cross-reactivity is present in solution, they don't cross-react at all. So the method of determining cross-reactivity is important. Again, I have two cross-reacting peptides which give the same binding constant on a plastic plate, and in solution one is 10^5 or 10^6 less strong. On that criterion, there is no cross-reactivity. If we put that same low cross-reacting peptide on a macrophage, the monoclonal antibody binds to it as well as to the peptide with a high binding constant. So the notion of cross-reactivity depends on the assay used.

Rothbard: In comparing the ability of anti-peptide with anti-protein antibodies to bind the protein we should consider the thermodynamics of the

process. I believe the limitations of the anti-peptide antibodies are due to the lack of a single, well-defined conformation in the peptides used as immunogens. Peptides lacking a defined conformation are capable of stimulating a large number of B cell clones, only a small fraction of which recognize the peptide in the conformation it adopts in the intact protein. How effective the peptide will be in eliciting a cross-reactive response will depend on the likelihood that either the peptide adopts the 'native' conformation, or the particular section of the protein has as much conformational freedom as the peptide. Consistent with this is the evidence that regions of high flexibility, for example the N- and C-termini of proteins, will obviously cross-react better than regions of rigid structure. The likelihood of the adoption of a particular conformation will be related to the energy requirements for adopting the structure.

There have been several reports of antibodies inducing conformational change (summarized in Habeeb 1977 and Celada 1983). Such induction of conformation is not without consequence. Thermodynamic arguments dictate that the energy necessary to induce a peptide to adopt a particular conformation will directly result in a lower affinity constant. If the energy requirements for induction are too severe, the B cell will not be stimulated to differentiate and the population of antibodies recognizing that particular conformation will not be present in the humoral response against the peptide.

One system for which I have data is a disulphide loop in pilin isolated from *Moraxella bovis*. The experiment I did is very similar to what Dr Sela and Dr Arnon did previously on lysozyme (Arnon et al 1971). I examined the ability of an antibody raised against the intact protein to recognize the amino acids composing the loop, depending on whether the cysteines were oxidized or reduced and alkylated. This experiment was possible because the loop is naturally highly immunogenic. As can be seen from Table 1, the antisera bound the oxidized loop twice as well as the reduced and alkylated material in a solid-phase binding assay.

Having shown that there are nearly identical amounts of peptide on the solid phase, I conclude that the differential binding is due to their different con-

TABLE 1 (Rothbard) Ability of antibodies elicited by intact Moraxella bovis pili to cross-react with peptides corresponding to the disulphide loop of pilin

Dilution of sera	*Bovine serum albumin (BSA) (c.p.m.)*	$\overset{\frown}{S\text{--}S}$ *--BSA (c.p.m.)*	*RS—SR--BSA (c.p.m.)*
1 : 50	785[a]	13904	7373
1 : 100	527	9529	2661
1 : 500	431	2611	956
1 : 1000	155	1386	506

[a] ^{125}I protein A bound to immunoglobulins in solid-phase binding assay

formations. I can relate this difference to free energy by using the differential binding as an equilibrium constant, k_{eq}, and using the following equation:

$$\Delta G = -RT \ln k_{eq}$$

$$k_{eq} = \frac{\text{counts bound to oxidized material}}{\text{counts bound to reduced and alkylated material}}$$

$$k_{eq} = 2$$

$$\Delta G = -0.4 \text{ kcal/mol}$$

As can be seen, a relatively small difference in free energy will result in a large difference in cross-reaction. An analysis of this logarithmic relationship between free energy and binding constants shows that when the differential binding equals ten, $\Delta G = -1.2$ kcal/mol and when $k_{eq} = 100$, the free energy difference is only -2.4 kcal/mol.

Therefore, if a peptide requires 2.5 kcal/mol to adopt the conformation that it has when part of the intact protein, then (i) when it is used as an antigen, antibodies against the protein directed at this sequence will bind the peptide with a binding constant two orders lower in affinity than the corresponding region of the protein, and (ii) when it is used as an immunogen, the anti-peptide antibodies will bind the intact protein with two logs lower affinity than they do the peptide. I believe that in order to elicit antibodies against relatively rigid regions of proteins, peptides must be designed to more closely resemble the intact proteins.

Williams: How many flexible pieces are there in these molecules?

Rothbard: The loop is quite big, 20 amino acids.

Williams: In that case, the difference in the weighted probability between the disulphide-bridged and the open-chain peptides due to conformational restriction should have been many thousand. The fact that you obtained only a difference of two suggests that the open form, through its lack of restriction, can match the receptor better than the closed form. The reason for this is relatively simple. The peptide loop in the closed form has a problem in presenting itself to the antibody in some conformations. The selection between open and closed forms then need not be an entropic problem, but is a ΔH problem. The free energy of binding equation has two terms, one to do with the binding energies and the other with entropies. The overall problem is that the closed loop had to get into some state which was energetically difficult for it, to make it sufficiently antigenic, but it was restricted in stereochemical terms. This disadvantage is partially offset by the gain of binding strength due to entropic restriction. The restrictions on the open form are in the opposite order.

Evan: On one occasion at least, we have isolated a monoclonal antibody that recognizes an intermediate state of naturation. This antibody recognized the c-*myc* protein only when it was on the Western immunoblotted nitrocellulose

filter. It did not recognize native material or totally denatured material. There may be something of interest which people should consider there.

The second point is that on several occasions now, polyclonal antisera, raised against a given synthetic peptide, have not been shown to have good cross-reacting activity against the intact protein. Yet on many of these occasions, a high proportion of the monoclonal antibodies against that peptide did cross-react. Perhaps we should be aware that in a solution of a monoclonal antibody the concentration of the binding site is enormously higher than the concentration of individual binding sites in any polyclonal serum, even one raised against a small synthetic peptide.

Sela: This is not of great help for making a synthetic vaccine, because you cannot induce people to make monoclonal antibodies as a result of immunization with vaccines.

Evan: That is right. The question is: how many different antibody combining sites are there in a polyclonal serum against a given synthetic peptide? If there are 1000, the concentration of the antibody combining site is a thousandth of the concentration of total antibody. Moreover, if each of those combining sites recognizes, or prefers, a specific sort of conformation of that flexing sequence in the intact protein, then the concentration of the antigen is a great deal lower as well. Has anyone asked what happens if we leave a polyclonal anti-peptide antibody for a long time with the intact protein containing the peptide antigen sequence? Will it bind then? Without this kind of information, it is difficult to compare monoclonal with polyclonal antibodies.

Rothbard: We have done that in solid-phase binding assays. Many anti-peptide antibodies which have high titres for the homologous peptide fail to cross-react with the corresponding protein on solid-phase assays when they are incubated together for only an hour. However, if the serum is allowed to incubate for long periods (24 hours) a strong signal is apparent when the plate is washed and subsequently incubated with either a second antibody or Protein A. The easiest explanation is that the protein on the plate is in equilibrium between a fully folded state and a series of partially unfolded states. The anti-peptide serum has a high concentration of high affinity antibodies that recognize the particular region in 'non-native' conformations. As the protein 'breathes', the antibodies can bind and subsequently prevent that region from refolding. Over a period of time the antibody–protein complex increases in concentration, resulting in a large signal after long incubation times. We know that this is not the result of non-specific binding because the signal specifically appears in the well containing the corresponding protein and not with alternative proteins.

Evan: This observation could explain, on its own, why there is greater success with monoclonal than polyclonal antibodies when one tries to produce anti-peptide antibodies which recognize intact proteins.

Klug: We have to distinguish the different purposes to which anti-peptide antibodies are put. Dr Evan is talking about *recognizing* a protein and it doesn't matter what state of folding it is in. Other people are concerned with making an effective neutralizing vaccine. The work on c-*myc* illustrates a case of simple recognition, which is what most cell biologists want. They are not concerned with success in vaccination. Each approach has its purpose, and I would like to hear from those making anti-peptide antibodies just what fraction of their antibodies are neutralizing. Of course, it may be too early to ask this. And what really is a neutralizing antibody?

Ada: That is precisely the point. It depends on the number of sites on the protein which are important for neutralization. That is, the number of sites which bind antibody and infection by the virus is prevented.

Lerner: Perhaps we are trying to describe a diversity system of the order of 10^{11}, one member at a time! Perhaps all we can say is that there are many possibilities.

Ada: If we are going to use peptides as the basis of a vaccine against, say, a virus, what is the frequency and efficiency with which antibodies to the peptides will react with the original protein or the virus, so that infection by the virus is prevented?

Sela: The numbers available are much too small, and the diversity of the system much too big, for a statistical analysis. In the case of one viral coat protein, we prepared two peptides of 20 amino acids each. There was no reason why one should be efficient in provoking neutralizing antibodies, and the other one not at all. In the case of the cholera toxin B subunit, out of six peptides, only two peptides led to the production of antibodies that neutralize the toxin.

Ada: Perhaps because in many cases we don't know the regions of the proteins that are important for neutralization, we should try to concentrate on the question of how efficiently antibodies to peptides will bind to the parent protein.

Stanworth: In our experience, if we put ε-chain peptides on KLH as carrier using glutaraldehyde, we can produce good anti-peptide antibodies which react with parent rat IgE. If we put the same peptides onto BSA using the same method, we still get good anti-peptide antibodies but they do not react with the parent Ig. You can't evoke here some stronger antigenicity of KLH than of BSA, because we are producing antibody responses to the peptide in both cases. So is there some difference in presentation, in terms of the relationship of the peptide to the carrier? Are we referring here to different carriers? Others have also had this problem, for instance in using BSA as a carrier, in relation to other carriers.

Ada: So there are other factors, apart from the peptide itself, that have an important role in determining the specificity of the anti-peptide response.

Lennox: If it is all so obvious, as Richard Lerner implies, what advice does he

give John Skehel about how to go about using peptides to make neutralizing antibodies? I think it is premature; you don't know what to tell him to do next.

Lerner: Yes. As Aaron Klug once said to me in discussing the anti-peptide antibody results: 'It is amazing that the dog speaks at all—we shouldn't expect an opera yet'.

Lachmann: In comparing antibodies for their degrees of cross-reactivity, one needs to know their affinity for various ligands in real units. This needs proper quantitative assays, rather than the excessive reliance on ELISA tests which give only arbitrarily quantitative data.

The proportion of monoclonal antibodies reactive to conformational or other linear sequences

Ada: May we now consider the question of the proportion of monoclonal antibodies that react to the various types of epitopes in polymeric proteins?

Williams: When we come to think about the different modes of antigenicity of a protein surface, we must distinguish the differences between the sequence and the conformational structure. The sequence gives you a linear pattern (A,B,C,D etc.). That is one possible source of antigenicity (type 1) that may be a recognition point for an antibody. Another (type 2) is a β-strand or helix, which is related to the sequence but the exposed surface may reveal amino acid A, then miss two or three (B,C), then reveal D or G on the surface. (I presume that some of the amino acids have to be on the surface to be recognized by the antibody at all.) Both of these recognitions depend on the sequence. When you are thinking of designing peptides, just from knowledge of the DNA, you have virtually no choice except to design from the linear sequence, but you can still design in these two ways. Once there is a three-dimensional structure, you have another possibility, to design antigenic peptides by deliberately looking at adjacent amino acids in space, not in sequence. This approach (type 3) is much more difficult to use, since it gives too many possibilities. People have suggested that perhaps the strongest antigenicity will arise from adjacent strands in space, one part of the antigenic site coming from one helix, and the other from a nearby strand, or helix.

What I find difficult is this. Running through all the discussions has been the motif of flexibility. If we think about flexibility in terms of these three possibilities for antigenicity, we see that they are very different. In the first type (sequence A,B,C,D is antigenic), flexibility would mean that groups A,B,C and D must all be revealed. Presumably, every now and again all four must appear together on the outside of the protein. This would need flexibility of the protein, because in protein structures it is not usual for all the side-chains in a

sequence of about five amino acids to appear on the surface in what we could call the most stable, or ground-state, protein structure.

Similarly, if the helix is to be antigenic (A,D,G is antigenic; type 2), flexibility is helpful. It is better if the protein can breath a bit, because then the surface of the helix is more revealed, without undoing the helix. The antigenicity remains in the secondary structure. However, tertiary structure is required by the third (type 3) kind of antigenicity, but tertiary structure is removed by flexibility. Flexibility opposes tertiary structure, because groups not in sequences move away from one another, so they lose close contact. Thus it would be hard to understand type 3 antigenicity in terms of flexibility. The more flexibility you have in the fold, the less the definition of such a site; whereas the flexibility in type 2 antigenic regions will reveal the site.

I believe that some exposure must be necessary for antigen–antibody binding to be obtained, but it may start by weak binding, when, in general, the first two types are more likely to be the ways for the antibody to search. In this case, too, it is right to start from the sequence and not to wait for the structure in order to probe antigenicity. It is also correct to look for hydrophilic sequences—that is, sequences on the surface. If type 3 antigenicity is common, so many possibilities come up, not connected to the flexibility argument in the same way, that it is difficult to see how to proceed.

Sela: Perhaps for the sake of symmetry you should add quaternary structure; in multi-subunit proteins where each subunit is completely native, even without any allosteric change you will often have some antibodies that are not adsorbed by the native subunit, and react only with the intact multi-subunit protein.

Williams: I accept that. I want also to point out that it is possible to create an antibody to almost anything if you let the antibody be weak enough, so I am not talking about very poor antibodies. It is pointless to say that everything is antigenic; the question is whether there are some sequences that are more antigenic than others.

Sela: The cross-reaction between a polymer of Pro-Gly-Pro and collagen is a typical example where it is not the detail of the sequence, but the helical shape, which controls the cross-reaction.

Ada: Mario Geysen perhaps has an instance of the third mode of antigenicity.

Geysen: I can at least say that our epitope (the foot-and-mouth disease virus mimotope) is not an example of type 1 or 2, because our defining monoclonal antibody was tested against all peptides, from six to 10 residues long. So we can exclude modes 1 and 2. However, I think that types 1 and 2 are the same, and types 3 and 4 are also the same. Perhaps the symmetry would be improved by removing the second mode, because a sequential determinant in fact includes spacer and contact residues; therefore to synthesize an example of the second

mode you still need to include spacers, which makes it indistinguishable from mode 1.

Sela: I would define an epitope as the juxtaposition of atoms in space, which in one case (sequential determinant) are contiguous within the sequence, whereas in the other cases the juxtaposition of the same number of atoms may come from amino acid residues which are quite far removed from each other in the primary sequence (conformational determinant).

Williams: I don't think the foot-and-mouth disease virus mimotope is a clean example of a non-contiguous antigenic site because of the way you searched in order to find a successful antigenic site. The question arises as to what your sequence is antigenic against, on the original protein; we do not know that.

Geysen: Then how do we interpret the absence of reaction of the monoclonal antibody with all the linear peptides based on the sequence of the viral proteins?

Anders: Would you classify any monoclonal that failed to react with an antigen when it was Western-blotted but reacted with the native protein in the same way?

Geysen: No! A Western-blotted antigen may fail to react with a monoclonal (intrinsically against a continuous epitope) by virtue of the protein being either in the wrong conformation, or absorbed on the cellulose nitrate support in an orientation which sterically precludes the interaction.

Klug: The fact is that there are many more examples of type 3 than of the others. If you look at an arbitrary patch in the surface of a protein, the chances of finding residues that are far apart in the sequence coming together is greater than residues contiguous in the sequence. Thus, on the whole, the monoclonals that are raised do *not* react with continuous peptides. *A priori*, you would expect type 3 recognition to be the most important case, but it is not reproducible with continuous peptides.

Crumpton: My experience with monoclonal antibodies against class I and class II histocompatibility antigens may be relevant here. Antibodies were selected on the basis that they react with the antigen as expressed on the cell surface. These monoclonal antibodies generally do not react by Western blotting against the reduced, SDS-treated antigen, although they immunoprecipitate very well the homologous antigen. Interestingly, some antibodies reacted by Western blotting with the SDS-treated antigen, provided it was not reduced, which argues for some retention of shape. Most other people's experience is the same. In other words, in this system monoclonal antibodies are rarely selected which recognize sequential determinants.

Sela: We ask all the time whether synthesized peptides react with antibodies to native proteins, but we haven't mentioned the in-between situation. If we take, for example, native ribonuclease or lysozyme, and see the extent to which antibodies prepared against the native protein react with the complete sequence of the same protein after denaturation, there will be essentially no cross-reaction; so why worry whether a peptide cross-reacts? The very fact that when a native protein is injected, the totality of the antibodies against it are not able to react with the open chain, when the disulphide bridges have been opened, means that all the antibodies are against a conformation.

Williams: This proves only that shape is required for antigenicity.

Altschuh: Again, it is a question of the relative affinity of the antibody for the peptide compared to the protein. The fact that a large molar excess of peptide over protein is needed in inhibition experiments means that anti-protein antibodies which have a reasonable affinity for the native protein have a low affinity for peptides. The serum may also contains a fraction of antibodies against the denatured antigen which bind well to peptides. If they bind weakly to the native protein, they will not be scored in competition experiments, but can be detected by direct binding assays with peptides. These antibodies are probably not significant in virus neutralization.

Lerner: With any method, what is the damnation of that method to one individual is its elegance to another. To me, the elegance of the anti-peptide technology is that it is site-specific. To those who want to neutralize all viruses, that is the damnation of the method. But unless you have a *general* site-specific technology, you probably won't have a lot of success in getting antibodies that neutralize viruses, because the sites that you need to hit on the surfaces of viruses to neutralize them are limited in number.

The site-specific nature of these antibodies is their essence. They may be used for perturbing protein structure, for localizing the products of genes, for carrying out structure–function studies, for domain mapping, for determining reading frames; and so on. The method is not however appropriate for generating broadly reactive sera.

Ada: There is increasing evidence that many antibodies are formed which recognize tertiary structures. If this is a general finding, what are the implications for antigen presentation and processing? Would such structures be seen only by B cells?

Lachmann: B cells see antigen in entirely native form so, whatever one's model for antigen processing, it cannot require that B cells look at processed antigen. It is known that there are hidden determinants inside IgG and C3 molecules to which antibodies are not made unless the native molecules are proteolysed before injection. These native proteins must therefore be seen by the B cell very much as they are.

Sela: When you inject the native protein, you can discover antibodies to proteolytic fragments, because of proteolysis by the body.

Lachmann: For plasma proteins like IgG and complement components (particularly C3), the immune response seems to look at the protein in the form in which it is given.

Humphrey: It is clear that B cells recognize the native configuration. Whether they can elicit T cell help by presenting that configuration is another matter, as we discussed earlier. From the repertoire of shapes of the Ig receptors on B cells can be selected some which bind to proteins in their native tertiary or even quaternary configuration (when a complex of two proteins is involved). What keeps the B cell clone going is another question.

Anders: Once again, it depends how you look. We initially detected expression of malaria antigens in *E. coli* by probing Western blots with antisera raised against β-galactosidase. We found that early bleeds gave clean Western blots, but antisera obtained after multiple boosts reacted with many fragments of β-galactosidase.

Ada: The message, though, seems to be that the immune system can cope with all these possibilities.

High and low affinity antibodies and their roles

Lerner: Dr Bill Jencks would say that the work of the cell is done by low affinity reactions!

Rothbard: As Dr Sela has stated, we must specify what our particular needs are. I have been attempting to raise antibodies with defined specificity with sufficiently high affinity for the intact protein to allow me to assign functional roles to particular structural domains. However, from our work with the gonococcus I am well aware that relatively low affinity antibodies can be useful in biological assays. I am also very interested in how peptides interact with T cells in biological protection.

Lachmann: It is the product of the concentration and the affinity of antibody which gives an effective signal. For a monoclonal antibody, at its very high concentration, a low affinity can be effective in giving a signal, whereas polyclonal antibody of the same affinity would be quite inactive. There is good evidence that animals that make large amounts of low affinity antibodies to certain viruses (e.g. lymphocytic choriomeningitis virus) *in vivo* are prone to develop immune complex disease (Soothill & Steward 1971). So, while all the cell's work may be done by low affinity antibodies, some of this work one doesn't wish to encounter in immunized animals or humans.

Williams: There is one big distinction between high and low affinity systems. A high affinity system is a better recognition system, but it doesn't come apart easily. So if there is a second stage in the biochemistry which depends on the system coming apart rapidly, tight binding in the first stage could be detrimental. If the antigen has to enter the B cell and some part of it has to be separated off, a low affinity system may be better because this process will be

quicker. The system might get stuck if it were a high affinity reaction. This is a well-known distinction between a kinetic and a thermodynamic equilibrium pathway. Bill Jencks is for this reason interested in low affinity biology. However, not all biological effects should be of low affinity or readily reversible. Some systems, like the blocking of proteases by trypsin inhibitors, must have binding constants as high as 10^{10} and the inhibitors are nearly perfect matches for the protease surfaces. High affinity stops systems, very often; low affinity in early steps allows a system to go in subsequent stages rapidly.

Anders: Would we accept that there may be circumstances, for example the malaria merozoite or sporozoite surface, where on-rates, and therefore affinity, could be critical in determining whether the antibody is effective?

Williams: Yes; the ratio of on-rate to off-rate is critical.

Lerner: Ordinarily we are talking about antibodies, which are replicating proteins, as it were, because there is a system to replicate them; whereas the antigen in a test tube (BSA, say) is a non-replicating protein. In a virus infection, though, you have a replicating antigen up against a replicating protein. A feature of a low affinity system is that you may use your antibodies over and over again.

Lachmann: The effector actions of the antibody will also be important here. Whether it is IgM or IgG and whether it fixes complement may be even more important than its affinity. The monoclonal antibody CAM-PATH-1, which kills T cells and prevents graft-versus-host disease (Waldmann et al 1984), is of low affinity (apparent affinity, $4–7 \times 10^7$ M^{-1}; G. Hale, personal communication), but because it is IgM and an excellent complement fixer, it is highly effective biologically.

Evan: It is also worth mentioning that with monoclonal antibodies in particular, the assay used to screen for positive antibodies has affinity parameters that one never bothers to think about, let alone define; yet these parameters may well limit the type of antibodies eventually selected.

Rothbard: Again, with vaccination, the concentration of antibodies in a particular site may be more critical than affinity. In mucosal immunity the problem is how do we particularly stimulate IgA. This is still poorly understood.

Lerner: That is a problem for any antigen, synthetic peptide or otherwise.

T-dependent and T-independent antigen presentation

Humphrey: There are certain instances where you can attach epitopes to a carrier, inject the conjugate in small amounts into human beings, and get a prolonged antibody response against the epitope. As I said, there are linear or non-linear polysaccharides which, if you put enough epitopes on, stimulate

thymus-independent antibody responses. At least in mice, rats and man, the antibodies appear to be made by a subset of B cells which is relatively sessile and resides in the marginal zone of the white pulp of the spleen. The evidence for this is that if you take the spleen out, the response is greatly diminished, and cannot be restored simply by supplying large numbers of spleen cells as a cell suspension (Amlot et al 1985). So the architecture of the spleen as well as the subset of B cells, and probably also the marginal zone macrophages as antigen-presenting cells, are all needed.

Polysaccharides such as Ficoll, hydroxyethyl starch or linear dextran with attached rhodamine, fluorescein or dinitrobenzyl groups and injected into mice or rats elicit antibody within one or two days. The response is maximal at five days but declines very slowly and persists for many weeks. The antibody is exclusively against the attached epitope. Perhaps, if peptides were attached to such carriers, or better still to lipopolysaccharide, you would get a prolonged antibody response. This would be worth testing, because one of the problems is how to keep the immunogen around. However, you get no detectable B memory cells by this method. We should remember the importance of the carrier, in respect of the kind of response we want to get. Victor Nussenzweig, for instance, wants to immunize with the circumsporozoite peptide on a carrier, and wants to be sure that if the mosquito injects sporozoites later on, the sporozoite antigen will stimulate the appropriate memory cells. I think that he should avoid using a carrier, if he can, or rather make the antigen become its own carrier by—perhaps—extending the peptide and polymerizing it.

Lachmann: Do you get any antibody other than IgM by this method?

Humphrey: Yes. In humans you also get IgG and IgA. In a rat it would be IgG2c mainly, and in the mouse IgG3. These classses of Ig are expressed on a sizeable proportion of the sessile population of spleen marginal zone B cells.

Ada: If you use a polymerized small epitope where you have essentially a similar repeating pattern, would you risk finding a significant proportion of an outbred human population who might respond poorly?

Humphrey: I suppose that one might. I think it would be wise to extend the peptides by using adjacent amino acid sequences, and perhaps to introduce some block to rapid degradation by peptidases—for example, a short polyproline sequence.

Sela: N.A. Mitchison showed that some (T,G)-A–L was compartmentalized, not in the lysosomal pockets in which it would be readily digested, but in another compartment in which it was preserved intact for many months.

Lerner: There is a problem with polymers. One tends to think that if one takes a seven-amino acid peptide and polymerizes it, this is the same as an equal number of multimers of the free peptide; but it isn't. Except for the two ends, each block of seven has the wrong neighbours, even though they are 'self' neighbours, and you greatly change what is going to happen. So, what experiment would you like us to try?

Humphrey: A simple experiment is to attach a peptide to a carrier such as Ficoll. I have tried this with luteinizing hormone-releasing hormone (LHRH), but I obtained only a small antibody response in mice. I perhaps did not attach enough epitopes, because I have since found that the antibody response is better when the epitope density is greater (unpublished); and I shall try again. It would probably be sensible to use a carrier such as lipopolysaccharide which can activate macrophages as well. Liposomes and micelles have already been mentioned (in my Introduction) as vehicles which increase antibody responses, and they are largely targeted towards macrophages. The thing to do is to try out different approaches, since we still do not really know what to do for the best!

Lachmann: Liposomes sound a very good idea. However, the extreme immunodeficiency in the acquired immunodeficiency syndrome (AIDS), which is believed to be due exclusively to failure of the T-helper system, suggests that without T cell help, a response adequate to provide immunity may be impossible. AIDS patients do not become hypogammaglobulinaemic and they do make antibodies, presumably largely by this T-independent system. But these antibodies fail to prevent all the AIDS-related infections.

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Synthetic IgE peptide vaccine for immunotherapy of allergy

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Abstract

An immunotherapeutic vaccine for allergy was produced by designing IgE-based synthetic peptide immunogens and selecting them for functional immunogenicity. The vaccine targets the binding site on IgE for the high affinity receptor FcεRI, by active immunization. The peptide target site on IgE heavy chain was selected from among the amino acid sequences for the Cε2, Cε3, and Cε4 domains. These were characterised by epitope mapping studies for cross-reactivity to IgE and functional antigenicity. A peptide, modified from positions 413–435 of a loop region of Cε3 and subjected to conformational constraint, elicited anti-IgE antibodies that blocked IgE-mediated histamine release. It was immunopotentiated by linkage to a promiscuous T helper site to produce a wholly synthetic chimaeric immunogen. This immunogen was shown to induce polyclonal site-specific anti-IgE antibodies that obstruct binding to FcεRI, inhibit histamine release by IgE-sensitised basophils, inhibit passive cutaneous anaphylaxis, and do not signal degranulation. Immunized dogs experienced significant reductions in total serum IgE.

Keywords: IgE; Peptide; Immunotherapy

1. Introduction

Immunoglobulin E (IgE) sensitises mast cells and basophils by binding to its high affinity receptor FcεRI, on the surface of those effector cells. Contact with antigen or anaphylactogenic anti-IgE antibodies causes the cross-linking of the bound IgE and the underlying FcεRI. The cross-linked receptors initiate a signal transduction cascade and rapid degranulation. The mast cells and basophils release stored histamine, followed by the synthesis and release of prostaglandins, leukotrienes, cytokines and other inflammatory mediators. These attract and activate inflammatory cells, produce the symptoms of allergy, and up-regulate the biosynthesis of IgE by B cells to promote heightened sensitivity. IgE-FcεRI interactions and the degranulation event are central to type 1 allergic reactions and to the development of atopic asthma [1–3]. The binding of IgE to the high affinity receptor has been a major target for intervention at the root level of type 1 allergic reactions [4].

The site on human IgE responsible for binding to FcεRI has been associated with the Cε2, Cε3, and Cε4 domains by binding inhibition studies involving recombinant IgE truncations [5,6], chimaeric IgE [7], site-directed mutagenized IgE [8,9], synthetic peptides corresponding to IgE Fc domains and antibodies induced by such peptides [10–14], and mimetope peptides [9,15]. These observations pointed to a highly conformational receptor binding site that has recently been solved by resolution of the crystal structure of a human IgE-FcεRI complex. This site has contact residues scattered among four surface loops that are asymmetrically dispersed between the two Cε3 domains of an IgE dimer and conformational scaffolding provided by Cε4 and the Cε2–Cε3 junctions [16]. Antibodies can obstruct this convoluted site by direct steric effect or induced conformational change.

Certain anti-IgE monoclonal antibodies whose recognition sites map to Cε3 interfere with the high affinity receptor binding site. These do not cross-link FcεRI-bound IgE and so they do not trigger degranulation and anaphylaxis [15,17,18]. Two of these antibodies are in clinical trial, and passive immunizations have provided desensitisation for patients with allergic rhinitis and allergic asthma [19–23]. An anti-IgE approach to immunotherapy by active immunization may have even greater promise than passive immunization due to cost effectiveness and potential

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for wider application. Rats sensitised to ovalbumin experienced suppression of skin reaction to the antigen following vaccination with a bacterially-expressed recombinant antigen comprising IgE domains Cε2–Cε3 coupled to a carrier protein [24] or with a Cε4 peptide coupled to a carrier protein [14]. However, those approaches to immunotherapy by active immunization were limited by (i) the risk that a large IgE fragment has the potential to generate anaphylactogenic antibodies, (ii) the inappropriateness of the target of the site-specific carrier-linked peptide, and (iii) low immunogenicity [25]. Here we sought to demonstrate the feasibility of an immunotherapeutic vaccine for allergy that uses wholly synthetic IgE peptide immunogens to target a site involved in FcεRI binding. Synthetic peptide immunogens were designed that generated high affinity, yet non-cross-linking (i.e. non-anaphylactogenic), site-specific anti-IgE. These antibodies blocked binding to the high affinity receptor, and were sufficiently immunogenic to evoke functional anti-dog IgE immune responses in dogs.

2. Materials and methods

2.1. Immunogen and antigen synthesis and processing

2.1.1. Peptide synthesis

Peptides were synthesized on a solid-phase support using an Applied Biosystems Peptide Synthesizer Model 430A and characterised as described [26]. Peptides having combinatorial library Th were prepared by providing a mixture of the desired amino acids at the specified positions. Combinatorial immunogens were characterised by size exclusion chromatography to a specification that requires 90% of the integrated area to exceed a mass threshold limit value, and by Edman degradation for N-terminal amino acid analysis.

2.1.2. Peptide modifications

Peptide-carrier protein conjugate immunogens were produced by chemical linkage of the target peptide to keyhole limpet hemocyanin (KLH) by MBS (*m*-maleimidobenzoyl-*N*-hydroxysuccinamide ester (Pierce, Rockford IL, USA). Liquid phase cyclization of peptide immunogens was accomplished by the formation of intramolecular disulphide bonds between substituent cysteines. The peptides were dissolved in water at 0.8 mg/ml, pH 3, DMSO was added to 1% (v/v) and NH_4OH was used to adjust to pH 7.5. The solution was incubated at ambient temperature in air and checked daily for 3 days by calorimetric assay using Ellman's reagent until disulphide bond formation was at least 90% complete.

2.1.3. Vaccine formulations

Peptide-based vaccines were water-in-oil single emulsions mixed 1:1 with Freund's complete/incomplete adjuvants or an adjuvant based on metabolizable oil, Montanide ISA 720 (Seppic, Fairfield NJ, USA).

2.2. In vivo analyses

2.2.1. Animals and immunizations

Duncan-Hartley guinea pigs were immunized intramuscularly with 100 μg of peptide immunogen or peptide-carrier conjugate on weeks 0, 3, 6, and 10. The first dose was administered with Freund's Complete Adjuvant and subsequent doses in Incomplete Freund's. Yorkshire-Landrace cross swine were immunized on a similar schedule with peptide vaccine in Montanide ISA 720. Balb/c mice were immunized subcutaneously on weeks 0, 3, and 6 with 20 μg of peptide immunogen in Freund's Complete for initial dose and Incomplete on subsequent boosts. Non-atopic beagles were immunized intramuscularly with peptide immunogen in Montanide ISA 720, on weeks 0, 3, and 7. Investigational procedures and animal care were in accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Academy of Sciences, 1996), and the guidelines of the Institutional Animal Care and Use Committees of United Biomedical Inc. and Covance Research Products Inc. (Denver PA and Kalamazoo MI, USA).

2.2.2. Measurement of anti-IgE antibodies

Anti-IgE peptide titres were determined by IgE peptide ELISA and cross-reactivities to human IgE by human IgE ELISA. Peptide ELISAs for determination of anti-IgE peptide reactivity were conducted in microtitre plates coated with the target antigen site peptide without the T helper site, as described [26,27]. For determination of anti-human IgE cross-reactivity, human IgE ELISAs were conducted in microtitre plates coated in a likewise fashion with a human IgE myeloma protein (American Biosystems Inc. cat. no. A113) at 5 μg/ml. Captured anti-peptide or anti-IgE antibodies were detected by horseradish peroxidase (HRP)-labelled anti-guinea pig IgG goat antibody or HRP-anti-swine IgG goat antibody. ELISA titres, expressed as $\log_{10}$ of reciprocal dilution, were calculated based on linear regression analysis of the absorbances, with cutoff A_{492} set at 0.5. This cutoff value was rigorous as the values for diluted normal guinea pig and swine control samples run with each assay were less than 0.15.

The anti-mouse IgE ELISA was as described for the anti-human IgE ELISA except that microtiter wells were coated with 1 μg/ml of mouse IgE anti-DNP monoclonal antibody SPE7 (Sigma, St. Louis MO), and HRP-goat anti-mouse IgG (Kirkegaard and Perry Laboratories, Gaithersburg MD) was used for detection of captured mouse IgG.

Anti-dog IgE responses to the trial immunization were determined by the peptide and IgE-based ELISAs as described above for the anti-human IgE assays, except that a hybridoma-derived dog IgE (Bethyl Laboratories, Montgomery TX, USA) was the solid-phase immunoabsorbant and bound dog IgG was detected by HRP-goat anti-dog IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

2.2.3. Measurement of serum IgE

2.2.3.1. Mouse serum IgE. IgE content of mouse sera was determined by a quantitative ELISA where mouse IgE was captured by coating the microtitre wells with 2 μg/ml of anti-mouse IgE mAb R-35–72 (Pharmingen, San Diego CA, USA), and the captured IgE was detected by HRP-sheep anti-mouse IgE (The Binding Site Inc., San Diego CA, USA).

2.2.3.2. Canine serum IgE. Immune dog sera were analyzed for total serum IgE, i.e. free IgE and IgE in immune complexes, by quantitative sandwich ELISA for IgE in heated sera [28]. Plates were coated with polyclonal anti-dog IgE, and captured IgE was detected by monoclonal antibodies specific for heat-stable IgE epitopes, as described [28].

2.3. In vitro analyses for functional immunogenicity

2.3.1. Inhibition of histamine release assay

IgG antibodies were purified from immune serum by Protein A affinity chromatography (ImmunoPure® Immobilized Recomb® Protein A, Pierce, Rockford IL, USA) and the eluted antibodies were prepared at a standard concentration of 8 mg/ml in 25 mM PIPES buffer, 0.15 M NaCl, pH 7.2. A control antibody preparation was prepared from sera of animals of the relevant species that were immunized with an irrelevant peptide immunogen. These antibodies were used in assays that measured the reduction in IgE-mediated sensitisation of human basophils. Human basophils were prepared from the venous blood of volunteers as described [29] except that the PAGCM buffer used to suspend the cells was made up with water containing 44% D_2O. The IgE used for sensitisation was human gp120-specific IgE (antibody to a peptide of the HIV glycoprotein gp120) [30] at 0.25 μg/ml. The allergen-specific IgE was preincubated with an equal volume of purified antibody at 8 mg/ml or dilution thereof, total volume 0.1 ml, for 15 min at 37 °C, prior to being added to the basophils. The antibody mixture was added to the cells and incubated for 20 min to allow for sensitisation of the cells by uncomplexed IgE (final concentration of the IgG antibody in the preincubation step was one-half the added concentration, and in the final incubation with cells, it was one-fourth the added concentration). The sensitised cells were then stimulated by addition of the allergen, gp120 peptide-ovalbumin (gp120P-OVA) [30] and the cells were incubated for 45 min. The cells were separated and the supernatant assayed for histamine content by an automated fluorimetric technique [31]. All reactions were performed in duplicate. The percentage of histamine release was calculated from the ratio of sample to total histamine after spontaneous release was subtracted from both. Results are expressed as per cent inhibition of histamine release, as determined from the ratio of histamine release by IgE preincubated with experimental antibody to histamine release by the IgE preincubated with control antibody of irrelevant specificity.

2.3.2. Inhibition of IgE binding by flow cytometry

IgG antibodies from immunized and control animals were purified by protein A affinity chromatography as described above. Human basophils were purified from leukapheresis cells as described previously [32]. The basophils (>99% purity) were sensitised with gp120-specific IgE (see above). IgG antibodies at various dilutions of the stock purified antibody at 8 mg/m were incubated with an equal volume of 1 μg/ml of gp120-specific IgE (for a total volume 0.1 ml) for 15 min at 37 °C, prior to being added to an equal volume of basophils in suspension (≈100,000 cells per condition). The antibody mixture was added to the cells and incubated for 20 min at 37 °C to allow for binding to the cells by uncomplexed IgE (final concentration of the IgG antibody in the preincubation step was one-half the added concentration, and in the final incubation with cells, it was one-fourth the added concentration). After two washes, the cells were labelled with mouse anti-idiotype antibody (specific for the gp120-specific IgE) [30], then secondary anti-mouse-phycoerythrin (PE) conjugate. The results were read by flow cytometry with controls being isotype matched mouse IgG. An additional control included cells that had not been sensitised with anti-gp120-specific IgE but were otherwise labelled with the anti-idiotype antibody and the anti-mouse-PE conjugate.

2.3.3. Flow cytometry

Flow cytometry was performed with a Becton-Dickinson cytometer. Forward and side scatter gates were set for the basophil population and PE fluorescence is calculated from the median of the distributions obtained.

2.3.4. Direct release

To determine whether the antibodies directly induced histamine release, Percoll separated leukocytes were challenged with either a standard goat polyclonal anti-IgE antibody (acting as a positive control) or with dilutions of the antibodies found to inhibit passive sensitisation. Leukocytes were challenged in PAGCM buffer for 45 min at 37 °C and supernatants harvested for analysis of histamine as described above.

2.4. Passive percutaneous anaphylaxis (PCA)

The 50 μl samples of diluted mouse serum from ovalbumin-sensitised mice were incubated with immune mouse sera or PBS controls for 1 h at 37° and then injected intradermally into the shaved backs of Sprague-Dawley retired breeder rats. After 24 h, PCA reactions were induced by intravenous injection of 1 mg of DNP-ovalbumin conjugate in 1% Evan's Blue dye. One hour later, the rats were euthanized and skinned. The DNP-Oa antigen had cross-linked receptor-bound mouse anti-Oa IgE on the rat mast cells. The cross-linking triggered degranulation, increased permeability of the Evans blue dye, and the appearance of blue zones on the underside of the rat skins proportional to the

extent of degranulation. However, wherever free IgE had been depleted by the site-specific murine anti-IgE, less was available to sensitise the rat mast cells and PCA reactions were suppressed. PCA reactions were evaluated by measuring the diameters of the blue zones on the undersides of the rat skins in two directions at right angles and taking the average.

3. Results

3.1. Identification of the IgE target site

Sites within the Cε2, Cε3, and Cε4 domains of human IgE were selected for synthesis as peptide immunogens based on previous observations and structural models that suggested potential effector sites [5,8,14]. These sites were also analyzed for surface-exposed loop structures deduced from models for the three-dimensional structure of IgE-FcεRI [8,16,33]. For peptides corresponding to predicted loop sites, disulphide-bonded loops were incorporated into the designs so as to stabilize the mobile peptides into conformations that resembled predicted IgE loop structures, and to maximize cross-reactivity between these designed target antigenic peptides and the native IgE molecule. Potential target antigenic sites were synthesized and made immunogenic either by chemical conjugation to KLH following solid-phase peptide synthesis, or by covalent attachment to promiscuous Th epitopes and other immunostimulatory sequences by continuous synthesis [25]. All 35 sites from IgE that were screened are shown in Fig. 1.

Candidate effector target sites were then identified by the preparation of hyperimmune sera in guinea pigs and testing of the antisera for reactivities to the target peptides and for cross-reactivities to human IgE. All peptide immunogens elicited anti-peptide responses. The kinetics of the antibody response show that anti-peptide ELISA reactivities appeared first, and for those peptides that evoked anti-IgE cross-reactivities, the cross-reactivity did not approach the levels of anti-peptide reactivity until week 8 post-initial immunization (data not shown). Antibodies from the 8 immune sera generated by the respective IgE antigens numbered in Table 1 had high cross-reactivities to human IgE. These antibodies were purified and evaluated for ability to inhibit the IgE-mediated sensitisation of human basophils by the in vitro assay for histamine release. Anti-peptide antibodies evoked by cyclized peptide immunogens 15b and 15c (Table 1) displayed strong cross-reactivity for IgE and most consistently displayed high inhibitory activity in the histamine release assay. This target epitope, at positions 413–435, corresponded to a segment of the Cε3 domain on a predicted surface-exposed loop.

The target site on peptide immunogens 15b and 15c was modified from that of the naturally occurring IgE sequence so as to provide a constrained loop structure. A cysteine residue was added to the N-terminus side of position 413 of human IgE ([34] for human sequence positions), the cysteine at 418 was replaced by serine, a cysteine was added at C-terminus side of position 435, and a disulphide bond was formed between the terminal cysteines to produce a cyclic structure. The target site peptide by itself was non-immunogenic. Extrinsic T cell help was provided

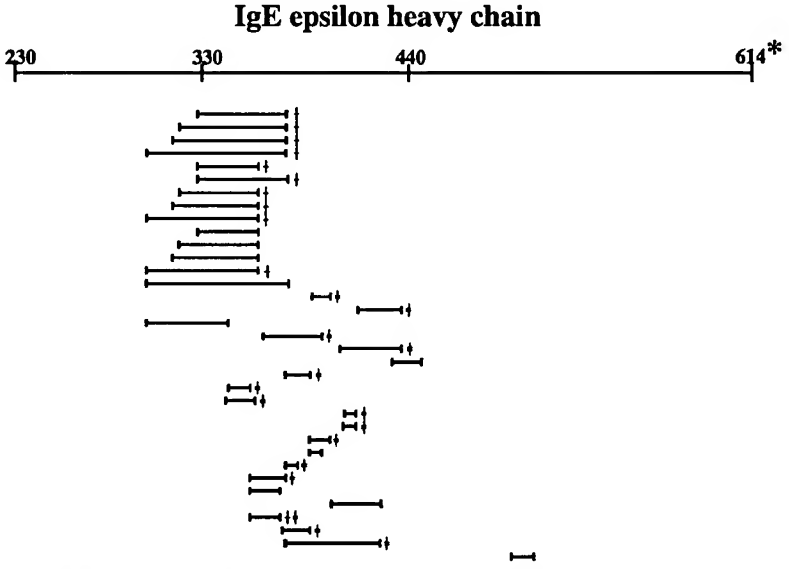

Fig. 1. Epitope mapping of functionally antigenic sites on human IgE epsilon chain.

Table 1
Evaluation of anti-IgE antibodies for inhibition of histamine release

IgE antigen no.	IgE antigen description	Immunogenic elements attached to IgE antigen	Cross-reactivity with human IgE (log_{10} titre)	% inhibition of histamine release[c] (%)
1	CH2/3 (328–376) ($C_{358} \rightarrow S$)[a]	KLH	3.66	0
2	CH2/3 (317–376) ($C_{358} \rightarrow S$)[a]	KLH	5.08	14
		UBITh®A-GG-	3.77	17 and 0
5	CH2/3 (328–362) ($C_{358} \rightarrow S$)[a]	KLH	4.40	0
6	CH2/3 (317–362) ($C_{358} \rightarrow S$)[a]	KLH	4.30	0
7	CH2/3 (313–362) ($C_{358} \rightarrow S$)[a]	KLH	3.92	6
8	CH2/3 (301–362) ($C_{358} \rightarrow S$)[a]	KLH	3.37	6
11	CH2/3 (313–356)	KLH	4.31	6
15	(C)CH3 (413–435) (C)[b] ($C_{418} \rightarrow S$)[a]	Syn Th(1, 2, 4)-GG	4.24	58[c] and 71[d,e]
		Inv-GG-Syn Th(1, 2, 4)-GG-	4.17	
20	(C)CH3 (374–382-(C)-383-385)[b]	HBs_{19-32}Th-GG	3.98	0
30	CH3 (399–424)	HBs_{19-32}Th-GG-	4.01	9 and 0
32	(C)CH3 (370–390) (C)[b]	HBs_{19-32}Th-GG-	3.45	0

[a] (C → S) serine substituted for cysteine.
[b] Cyclized peptide (C) cysteine introduced into native sequence for cyclization.
[c] Histamine release inhibition by antibodies to peptides, purified from serum collected at week 8, except as otherwise noted.
[d] Results are shown for pooled anti-15b and anti-15c IgG's.
[e] Histamine release inhibition by antibodies to peptides, collected at week 12.

by linkage to a synthetic UBITh® epitope "Syn Th (1, 2, 4)", a palindromic sequence derived from hepatitis B virus [25,35] or later by the artificial combinatorial T helper site derived from measles virus "UBITh®A" (shown in Fig. 2). UBITh®A has been shown to be promiscuously immunogenic across species [35]. T cell help was also imparted to a series of peptides by a T helper epitope at positions 19–33 of hepatitis B virus surface antigen [36].

3.2. *In vitro characterisation of functional immunogenicity*

A peptide immunogen having UBITh®A, the modified human 413–435 Cε3 target site, and an (ε-*N*) Lysine linker was synthesized (Fig. 2) and used to immunize a swine. This UBITh® IgE immunogen, Ap2878, was formulated with Montanide ISA 720 and 300 μg doses were administered intramuscularly to pig no. 183 on weeks 0, 3, 6, and 10. IgG was purified by protein A affinity chromatography from serum collected on week 12 and control IgG was prepared from a pig immunized with an irrelevant peptide immunogen. Purified human basophils were used to detect inhibition of sensitisation with IgE. Fluorescence flow cytometry data are compared (Fig. 3) for the antibody preparation from swine no. 183, and the control antibody, for inhibition of binding. Fig. 4A shows a titration of the 8 mg/ml purified antibody stock used to inhibit sensitisation, showing an IC_{50} of 1/250 (concentration in the cell suspension). The polyclonal antibody purified from swine no. 183 immune serum exhibited inhibitory activity for human IgE binding. In Fig. 4B, bar graphs are shown for the indirect assay of sensitisation of basophils by measuring subsequent antigen-driven histamine release. Measurement by flow cytometry or by histamine release yielded essentially equivalent results. For the basophils sensitised in the presence of swine no. 183 antibody, their response to goat polyclonal anti-IgE antibody was the same as for cells sensitised in the presence of control antibody, indicating that the vaccine antibodies had no non-specific effects on basophil function and did not desensitise the general IgE-mediated response (data not shown).

3.3. *Non-anaphylactogenicity*

The swine no. 183 antibody preparation and several other antibodies from guinea pigs that had been hyperimmunized with UBITh® human IgE immunogen (Fig. 2) were used to directly challenge human basophils. Histamine release by the vaccine-induced anti-IgE specimens did not exceed the level of spontaneous release by the basophils of the donors tested, indicating that the site-specific anti-IgE

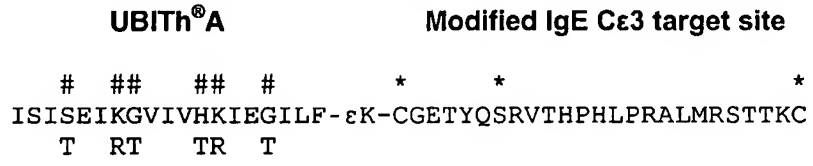

Fig. 2. UBITh®A immunogen for human IgE. T cell help is provided by the UBITh®A site shown, with combinatorial positions marked by (#). The Cε3 target antigenic site, linked to the T helper epitope through the ε-Lysine linker, has been modified from the native human sequence at the positions marked by (*).

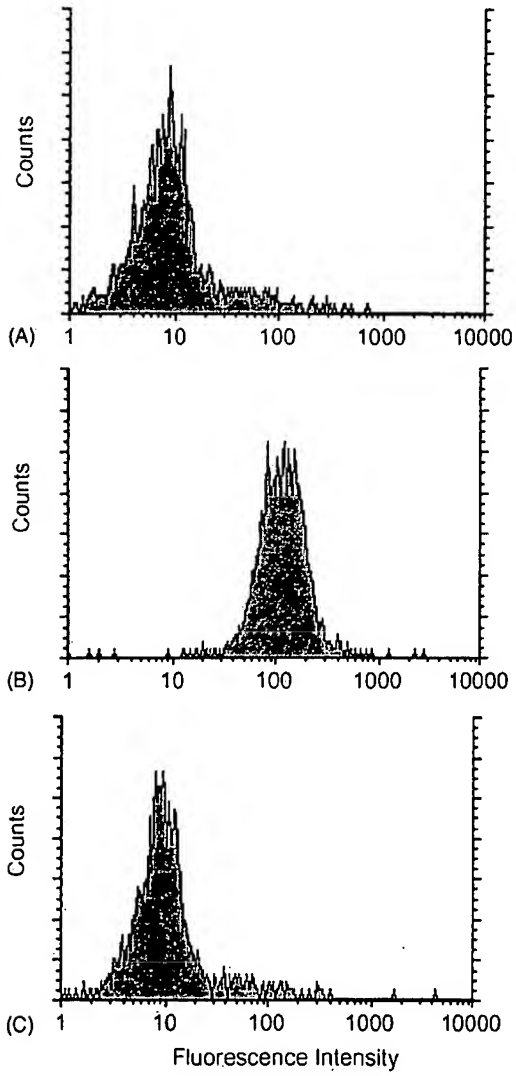

Fig. 3. Inhibition of human basophil sensitisation with vaccine-elicited anti-IgE antibodies. Purified basophils were sensitised in the presence or absence of vaccine-elicited anti-IgE antibodies and the effect of sensitisation assessed by flow cytometry. Panel A: cells not sensitised with gp120-specific IgE but labelled with the primary and secondary antibodies for flow cytometry, panel B: cells sensitised in the presence of control vaccine-elicited antibodies, and panel C: basophils sensitised in the presence of vaccine-elicited antibodies (swine no.183) at a concentration of 2 mg/ml (see text).

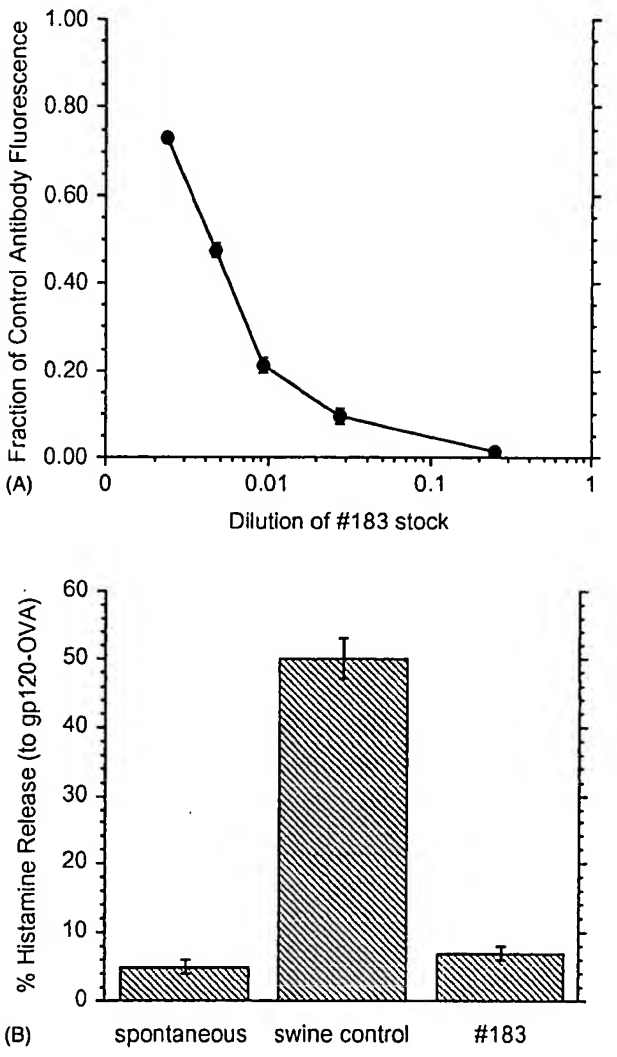

Fig. 4. Inhibition of human basophil sensitisation assessed by direct and indirect measures. Panel A shows the average of 2 experiments in which several concentrations of swine no. 183 antibody were tested for inhibition of sensitisation as detected by flow cytometry (as performed in the experiment described in Fig. 3). The data is calculated from the medians of the cytometric histograms. The data is expressed as a fraction of the median fluorescence (minus background) for cells sensitised with gp120-specific IgE in the presence of control swine antibody. The abscissa is the dilution of no. 183 as it is in the presence of cells (see Section 2). Panel B shows the results for experiments where the level of sensitisation was assessed by subsequently stimulating the basophils with specific antigen (gp120-OVA in these studies). In this example, sensitisation was tested in the presence of swine no. 183 antibody (or control Ab) at a concentration of 1:4 of the stock Ab.

antibodies did not cross-link receptor-bound IgE and were non-anaphylactogenic. In particular, swine no. 183 antibody, which was characterised in Fig. 3 for inhibition of sensitisation, induced no release at a final concentration of 1:10 (of 8 mg/ml stock) from basophils obtained from four donors which otherwise released 33 ± 14% to a positive goat polyclonal anti-IgE antibody.

3.4. Functional immunogenicity by passive cutaneous anaphylaxis assay

To study the effect of immunization by a UBITh® IgE immunogen on an IgE-mediated inflammatory reaction, an antibody response was elicited by immunizing mice with a UBITh® peptide immunogen having the autologous murine IgE-Cε3 target antigenic site (modified from the mouse sequence shown in Table 2). The resulting mouse antiserum was then used to suppress the passive cutaneous anaphylaxis (PCA) triggered by the cross-linking of mouse IgE bound to rat mast cells.

Table 2
Homologous IgE Cε3 target sequences

Human ε	GETYQCRVTHPHLPRALMRSTTKTSGPR
Chimp ε	GETYQCRVTHPHLPRALVRSTTKTSGPR
Rat ε	GEGYQCRVDHPHFPKPIVRSITKAPGKR
Mouse ε	GYGYQCIVDHPDFPKPIVRSITKTPGQR
Dog ε	GETYYCRVTHPHLPKDIVRSIAKAPGKR
Cat ε	GETYQCKVTHPDLPKDIVRSIAKAPGRR
Horse ε	GETYKCTVSHPDLPREVVRSIAKAPGKR
Pig ε	GETYYCNVTHPDLPKPILRSISKGPGKR
Goat ε	GETYYCKVSHGDLPKDIQRSISKDVGKR

Balb/c mice were immunized subcutaneously with 20 μg of the UBITh® mouse IgE peptide on weeks 0, 3, and 6. On week 8, mouse sera were collected and evaluated for cross-reactivity to IgE by the mouse IgE ELISA. Thirteen out of 20 immunized mice had cross-reactive antibodies against mouse IgE. Sera was pooled from seven mice showing ELISA titres against mouse IgE of $\geq \log_{10} 2.3$ for use as the site-specific anti-IgE. Another group of 10 balb/c mice was sensitised to ovalbumin (Oa) (1 μg per 0.2 ml in PBS on 0.4% alum, intraperitoneal injection). IgE content of the mouse sera was measured at day 20 by the ELISA for detection of murine IgE. Out of the 10 Oa-immunized mice, 7 had appreciable IgE responses of titre $\geq \log_{10} 1.6$. These sera were pooled for use as the anti-Oa IgE working stock.

The anti-Oa IgE serum pool was serially diluted 1:62, 1:124 and 1:248 into PBS, for use in PCA reactions in rats. These dilutions were later further diluted by pre-incubations with equal volumes of the site-specific anti-IgE serum pool. Thus, final dilutions of the PCA reactants was 1:124, 1:248, and 1:496 for mouse IgE serum and 1:2 for mouse anti-IgE serum. Control dilutions of IgE were prepared having only PBS as diluent. The IgE dilutions, with and without anti-IgE serum, were incubated for 1 h at 37° and 50 μl of each was taken for evaluation by PCA reaction in Sprague-Dawley rats.

The 50 μl samples of mouse IgE, pre-incubated with mouse anti-IgE serum or PBS control, were injected intradermally into the shaved back of rats in a pattern that was a set of two rows of four injections. The rows were a row of three controls of IgE diluted 1:124, 1:248, and 1:496 in PBS only, in parallel with a row of the serially diluted IgE incubated with the site-specific anti-IgE. The fourth injection of each row was PBS only, as a control for tissue trauma. The pattern was duplicated on two rats. After 24 h, PCA reactions were induced by intravenous injection of DNP-Oa conjugate in 1% Evans blue dye and results were observed 1 h later. The rats differed by their inherent sensitivities to the mouse IgE so that control and anti-IgE inhibited PCA reactions are compared independently for each rat (Fig. 5). Mouse IgE-mediated PCA reactions were inhibited in both rats by the murine antiserum with specificity for the mouse IgE target site.

3.5. *In vivo functional immunogenicity in dogs*

Non-atopic beagles were immunized with the UBITh® canine IgE peptide immunogen. This peptide has the canine IgE target antigenic sequence (modified from the dog sequence of Table 2) linked to the UBITh®A T helper site. The dogs were divided into five groups, four dogs per group. Groups 1–4 were immunized with 2000, 400, 100,

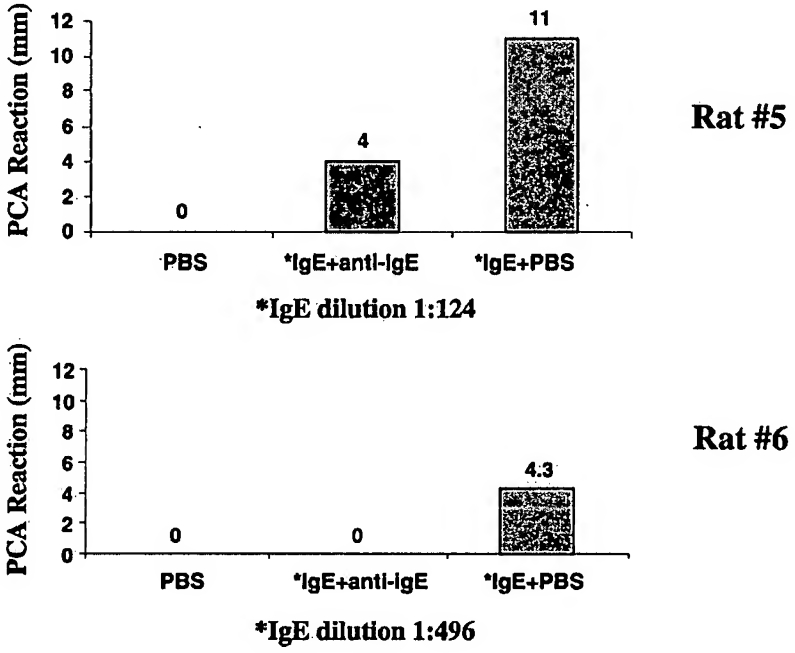

Fig. 5. Passive percutaneous anaphylaxis assays. Murine IgE pre-incubated with murine anti-IgE antibodies were used for the in vivo sensitisation of rat mast cells. Inhibition of sensitisation evaluated by extent of allergen-induced anaphylaxis.

or 25 μg, respectively, of the UBITh® canine IgE peptide in ISA 720, at 0 and 3 weeks post-initial immunization (wpi). At 7 wpi, the dogs of groups 1–3 were boosted with 100 μg while the group 4 dogs were given 25 μg. A fifth group was immunized with an irrelevant UBITh® vaccine for control. Dog serum was collected and analyzed for an anti-IgE response by anti-dog IgE ELISA. All four dogs of group 3, given the 100 μg doses on weeks 0, 3, and 7, achieved peak responses over background level that were sustained through week 9 (Fig. 6). For groups 1 and 2, three out of the four dogs of each group were responsive though less so than group 3, while the dogs of group 4, given 25 μg doses, were only minimally responsive above the control group (data not shown). The results indicate that 100 μg was the optimum immunogenic dose for the beagles.

The sera collected from two responsive dogs from each of groups 1–3 and a dog from control group 5 were assayed for total IgE content, including free IgE and IgE within anti-IgE/IgE immune complexes, by the quantitative assay on heated serum [28]. Serum IgE for responsive dogs from group 1 (nos. 27 and 28), group 2 (nos. 30 and 31), group 3 (nos. 32 and 33) and control dog no. 01 from group 5 are shown in Fig. 7 for weeks 0, 5 and 9. The range of initial IgE levels varied among these normal dogs, but the relative

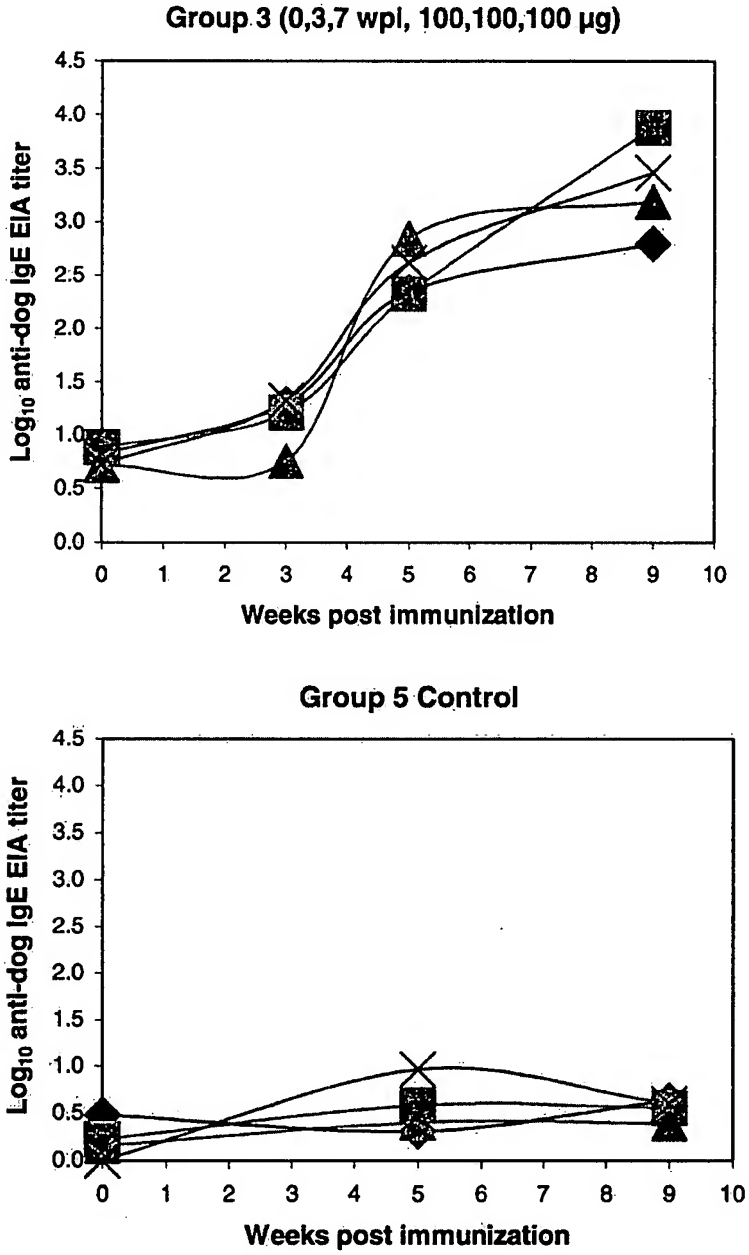

Fig. 6. Anti-IgE IgG responses in dogs to the dog IgE target immunogen.

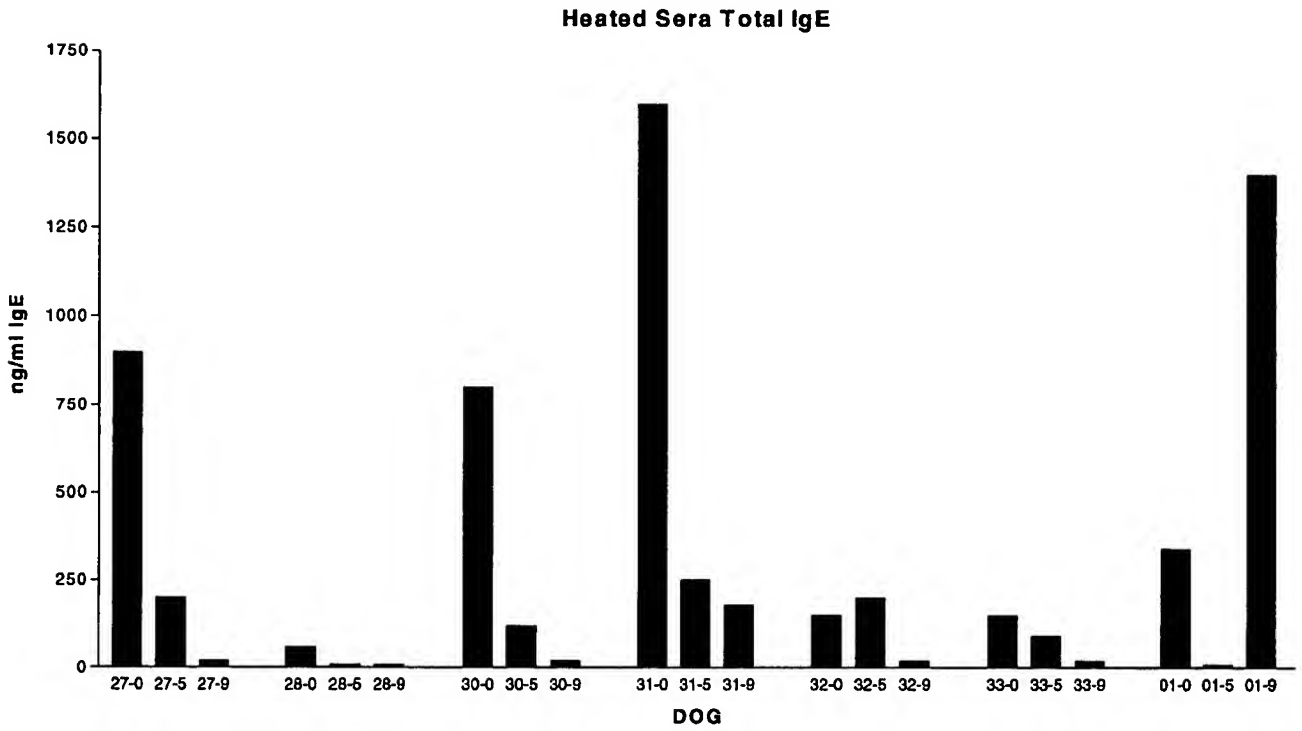

Fig. 7. Total IgE in heated dog sera, both free and complexed, was determined for dogs at weeks 0, 5, and 9 post-initial immunization. Dog nos. 27 and 28 were given initial dose 2000 μg, nos. 30 and 31 were given 400 μg, nos. 32 and 33, 100 μg, and no. 01 received placebo.

levels of total IgE in circulation declined substantially during the course of the trial immunizations. Dog no. 31, with the lowest anti-IgE (reciprocal titre $\log_{10}$ 1.8) at week 9 and the highest IgE at 0 week of the group 1–3 dogs tested, had total IgE at week 9 reduced by close to 10-fold from week 0.

4. Discussion

An IgE-directed immunotherapeutic approach aims to prevent initiation of the allergic cascade, and unlike classic desensitisation therapy, it is not allergen-specific. It can be effective for all IgE-mediated allergic reactions with no need to identify the offending antigen (e.g. pollens, dust mite allergen, molds). Proof-of-concept for an anti-IgE vaccine has already been established by clinical trials using passive immunization with non-anaphylactogenic anti-IgE monoclonal antibodies [19–23]. We have provided for a chemically-defined immunogen that elicits a polyclonal site-specific anti-IgE response. The IgE target antigenic site was immunopotentiated by linkage to a combinatorial T helper epitope derived from measles virus, UBITh®A. This T help was promiscuous across species, in guinea pigs, mice, swine, dogs, and elsewhere in baboons [35] and goats and cattle (to be submitted), when combined with various self and foreign B cell epitopes taken from Luteinizing Hormone Releasing Hormone (LHRH or GnRH), domain 1 of CD4, an N-terminal fragment of â-amyloid protein, somatostatin, and foot-and-mouth disease virus, in addition to IgE. Tolerance to the self target IgE antigen was overcome in dogs and to a lesser extent in mice by the artificial UBITh®A T helper epitope as shown by the production of auto IgG anti-IgE antibodies. The artificial T helper epitope also provided for antibody maturation. The kinetics of the antibody response in guinea pigs and swine (data not shown) showed that by week 8, anti-IgE cross-reactivities approached the anti-peptide reactivities. We concluded that as the anti-peptide antibody response matures, higher affinity antibodies are expressed and this secondary anti-peptide response becomes cross-reactive with the target protein.

Like the therapeutic monoclonal antibodies, the site-specific vaccine-induced antibodies blocked the binding of IgE to the high affinity receptor FcεRI, prevented anaphylactic reactions, and did not by themselves cross-link IgE and signal degranulation. Like the passive immunization trials performed in mice [37], the active immunization of dogs resulted in reductions in serum IgE. These reductions were correlated to the anti-IgE response, except in those normal dogs that had low serum IgE at the start of the vaccine trial (data not shown). However, the reduction in total serum IgE may be a species effect since trials in humans have yet to find a reduction in total IgE levels even after 1 year of treatment [21–23] (and unpublished reports).

Homologous target peptide sequences for human, chimp, rat, mouse, dog, cat, horse, pig, and goat IgE are compared in Table 2. In this case, homologous sequences did result

in homologous function since the target sequences from human, murine, and canine epsilon chains all exhibited the same functional antigenicity of blocking the binding of IgE to the high affinity receptor. This broad effect is predictive of efficacy for immunotherapeutic allergy vaccines in multiple species, including humans and dogs.

The vaccine-elicited antibodies blocked the sensitisation of mast cells and basophils by free IgE. However, this action mode alone cannot by itself provide the pharmacological mechanism for therapeutic efficacy by the antibodies or a vaccine. Even if free IgE were neutralized to 99% by the anti-IgE, the therapy still would fail because the few remaining IgE molecules would be sufficient to sensitise the mast cells and basophils [29]. This would be a more serious problem in dogs since levels of IgE are relatively high compared to humans [28]. However, an anti-IgE approach has the potential to provide therapeutic efficacy through other actions. Human basophil studies have shown that FcεRI expression is regulated by the level of free IgE, so that reduced levels of free IgE should lead to lower densities of FcεRI on basophils and mast cells and lowered sensitivities [19,38,39]. And, anti-IgE may lead to the down-regulation of IgE production by eliminating or down-regulating IgE-expressing B cells, perhaps by cross-linking membrane-bound IgE and causing apoptosis or anergy [37,40] or perhaps by complement-mediated and cell-mediated cytolysis [41]. It has been difficult to demonstrate reduction of IgE-producing B cells in the clinical trials with monoclonal antibodies, but other in vivo and in vitro experimental results are supportive of an anti-IgE-expressing B cell mechanism [18]. Whatever the underlying mechanism, the reduction of IgE levels in the dog trials suggests that tolerance for IgE can be broken and that the appearance of anti-IgE has a dramatic effect on at least one aspect of the immune response. Future studies will be needed to determine whether this characteristic will be found in primates.

Acknowledgements

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